Article title	Erinacin, an antihaemorrhagic factor from the European hedgehog, Erinaceus europaeus
Article identifier	0041010197002178
Authors	Mebs_D Omori-Satoh_T Yamakawa_Y Nagaoka_Y
Journal title	Toxicon
ISSN	0041-0101
Publisher	Pergamon
Year of publication	1996
Volume	34
Issue	11-12
Supplement	0
Page range	1313-1316
Number of pages	4
User name	Adonis
Cost centre	Development
PCC	$20.00
Date and time	Tuesday, June 24, 2003 10:43:21 AM

Pergamon

PII: S0041-0101(96)00104-3

Toxicon, Vol. 34, No. 11/12, pp. 1313–1316, 1996

0041-0101/96 $15.00 + 0.00

ERINACIN, AN ANTIHAEMORRHAGIC FACTOR FROM THE EUROPEAN HEDGEHOG, *ERINACEUS EUROPAEUS*

D. MEBS,[1] T. OMORI-SATOH,[2] Y. YAMAKAWA[2] and Y. NAGAOKA[2]

[1]Zentrum der Rechtsmedizin, University of Frankfurt, Kennedyallee 104, D-60596, Frankfurt, Germany; and [2]National Institute of Health, Toyama 1-23-1, Shinjuku, F162, Tokyo, Japan

(*Received* 14 *September* 1995; *accepted* 23 *October* 1995)

D. Mebs, T. Omori-Satoh, Y. Yamakawa and Y. Nagaoka. Erinacin, an antihaemorrhagic factor from the European hedgehog, *Erinaceus europaeus*. *Toxicon* **34**, 1313–1316, 1996.—An antihaemorrhagic factor named erinacin was purified from the skeletal muscle extract of the European hedgehog, *Erinaceus europaeus*, by ammonium sulfate precipitation followed by various steps of ion-exchange (DEAE-cellulose), absorption chromatography (hydroxylapatite), and gel filtration (cellofine gel). A 625-fold purification was achieved with an overall yield of 19% antihaemorrhagic activity. The protein effectively inhibited the activity of *Bothrops jararaca* venom haemorrhagin and did not inhibit the enzymatic activity of trypsin and chymotrypsin. Erinacin is a large molecule (about 1,000,000 mol. wt). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed the presence of two subunits: one with an apparent mol. wt of 35,000 forming a larger subunit (350,000) by cross-linking with disulfide bridges, and a second with a mol. wt of 39,000 without disulfides. Dissociation of erinacin into its subunits resulted in complete loss of its antihaemorrhagic activity. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Proteins inhibiting the activity of snake venom haemorrhagins have been detected in the blood of venomous and non-venomous snakes, as well as various mammals (Domont *et al.*, 1991). From the plasma of the European hedgehog, which occasionally feeds on vipers (*Vipera* sp.) and which seems to be resistant to their venoms, an antihaemorrhagic factor also exhibiting proteinase inhibitor activity has been isolated and characterized to be a β-macroglobulin with a mol. wt of 700,000 (De Wit and Weström, 1987a, b). Similar activity inhibiting haemorrhagins in several venoms from snakes such as *Bitis* sp., *Bothrops asper* and *Bothrops jararaca*, *Vipera berus* and *Vipera latastei* has been found in skeletal muscle extracts of the hedgehog (Omori-Satoh *et al.*, 1994).

This report describes the purification of this antihaemorrhagic factor, named erinacin, and its subunit composition.

MATERIALS AND METHODS

Hedgehogs (*Erinaceus europaeus*) which were found dead on roads (killed 5–6 hr before) around Frankfurt (Germany) were used. Muscle samples were excised, homogenized in physiological saline using a blender and centrifuged, and the supernatant was lyophilized. The minimum haemorrhagic dose (MHD) producing a haemorrhagic spot of 10 mm diameter in depilated rabbit skin was assayed according to the method of Kondo *et al.* (1960) using crude venom from *B. jararaca* (MHD 0.082 μg). A dose of 25 MHD was incubated with varying amounts of muscle extracts or its fractions in 0.5 ml (0.01 M phosphate-buffered saline, pH 7.0) at room temperature for 1 hr, and 0.2 ml of the mixture (10 MHD of venom) was tested; the antihaemorrhagic dose was defined as the amount of material (mg) inhibiting 10 MHD of the venom.

RESULTS AND DISCUSSION

Purification of the antihaemorrhagic factor erinacin

The lyophilized muscle extract was dissolved in 0.05 M phosphate buffer, pH 7.4, containing 0.15 M NaCl, and centrifuged, and 50 ml of saturated ammonium sulfate solution was added to the supernatant (100 ml). The precipitate, dissolved in 0.05 M Tris–HCl buffer, pH 8.5, and dialysed against the same buffer, was applied to a DEAE-cellulofine column eluted with a linear gradient of NaCl (0.3 M). The fractions exhibiting antihaemorrhagic activity were further separated on a hydroxylapatite column eluted with a linear gradient of phosphate buffer (0.01–0.3 M, pH 7.0). Final purification was achieved by gel filtration on a cellufine GCL-2000 superfine column (Fig. 1). Erinacin was obtained with an overall yield of 19% of antihaemorrhagic activity, purified 625-fold. The preparation was homogeneous in polyacrylamide gel electrophoresis (PAGE) and gel high-performance liquid chromatography (HPLC). The molecule is stable, and no loss of activity was observed when stored in solution at 4 C, which is in contrast to observations on the plasma antihaemorrhagic factor described by De Wit and Weström (1987b).

Molecular weight and subunit composition of erinacin

In gel filtration erinacin behaved as a large molecule eluting slightly faster than immunoglobulin M (900,000 mol. wt), suggesting a mol. wt of about 1,000,000. In sodium

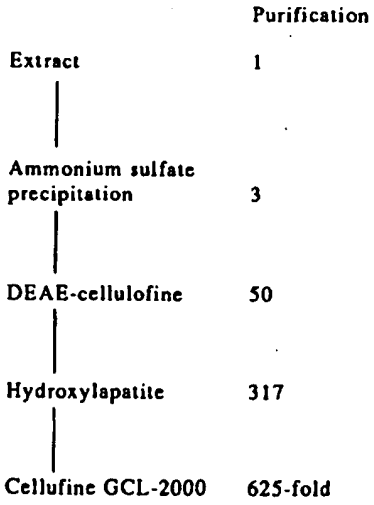

Fig. 1. Purification of the antihaemorrhagic factor erinacin from skeletal muscle extract of the European hedgehog, *Erinaceus europaeus*.

dodecyl sulfate (SDS)–PAGE under reducing conditions (marcaptoethanol) two bands corresponding to 35,000 and 39,000 mol. wt, respectively, occurred, indicating that erinacin consists of two polypeptide subunits.

After exposure to 6 M guanidine–HCl the protein dissociated into two components separated by gel HPLC: S1 and S2, in a ratio of 1:2 (protein amount). This treatment caused complete loss of antihaemorrhagic activity, which was not restored by combining the fractions. Whereas the mol. wt of S2 was calculated to be about 350,000, that of S1 was estimated to be 39,000. By reducing the disulfide bonds in S2, components exhibiting a mol. wt of 35,000 were obtained in SDS–PAGE.

From these data it is concluded that erinacin is a complex of various subunits: two identical molecules (S2) consisting of ten subunits (each of mol. wt 35,000), which are cross-linked by disulfide bridges, and ten smaller subunits (S1, mol. wt 39,000). Altogether, S1 (390,000) and 2 × S2 (350,000) amount to a total mol. wt of 1,090,000. The antihaemorrhagic factor from the hedgehog plasma, characterized as β-macroglobulin, had also been found to have a mol. wt of 700,000 and was supposed to consist of two types of subunits with mol. wts of 34,000 and 39,000 (De Wit and Weström, 1987b).

Inhibition of proteinase activity

When erinacin was preincubated with TPCK-trypsin or chymotrypsin in a molar ratio of 1:1, inhibition of esterolytic activity of both enzymes was observed (TAME and BTEE as substrates).

CONCLUSION

Erinacin is an exceptionally large molecule with a complex subunit composition. It is not related to other antihaemorrhagic factors isolated so far, such as from snake serum (*Trimeresurus flaviviridis*), which belongs to the cystatins (Yamakawa and Omori-Satoh, 1992), from opossum serum (*Didelphis virginiana*), a protein similar to human α1B-glycoprotein (Catanese and Kress, 1993), or from mongoose serum (*Herpestes edwardsii*), which exhibits sequence homology to oprin (Qi *et al.*, 1995). However, it may be related to the antihaemorrhagic factor found in the plasma of the hedgehog. Studies are in progress to elucidate the structural basis of the antihaemorrhagic activity of this peculiar molecule.

REFERENCES

Catanese, J. J. and Kress, L. F. (1993) Opossum serum α_1-proteinase inhibitor: purification, linear sequence, and resistance to inactivation by rattlesnake venom metalloproteinases. *Biochemistry* **32**, 509–515.

De Wit, C. A. and Weström, B. R. (1987a) Venom resistance in the hedgehog, *Erinaceus europaeus*: purification and identification of macroglobulin inhibitors as plasma antihaemorrhagic factor. *Toxicon* **25**, 315–323.

De Wit, C. A. and Weström, B. R. (1987b) Purification and characterization of α_2-, α_2-β- and β-macroglobulin inhibitors in the hedgehog, *Erinaceus europaeus*: β-macroglobulin identified as the plasma antihaemorrhagic factor. *Toxicon* **25**, 1209–1219.

Domont, G. B., Perales, J. and Moussatché, H. (1991) Natural anti-snake venom proteins. *Toxicon* **29**, 1183–1194.

Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. *Jpn. J. med. Sci. Biol.* **13**, 43–51.

Omori-Satoh, T., Nagaoka, Y. and Mebs, D. (1994) Muscle extract of hedgehog, *Erinaceus europaeus*, inhibits hemorrhagic activity of snake venoms. *Toxicon* **32**, 1279–1281.

Qi, Z.-Q., Yonaha, K., Tomihara, Y. and Toyama, S. (1995) Isolation of peptides homologous to domains of human α1B-glycoprotein from a mongoose antihemorrhagic factor. *Toxicon* **33**, 241–245.

Yamakawa, Y. and Omori-Satoh, T. (1992) Primary structure of the antihemorrhagic factor in serum of the Japanese Habu: a snake venom metalloproteinase inhibitor with a double-headed cystatin domain. *J. Biochem.* **112**, 583–589.